BSSD 2019 Performance Metric Q1

Goal: Develop metagenomics approaches to assess the functioning of microbial communities in the environment.

Q1 Target: Describe progress in the use of metagenomic techniques to detect and describe the composition of microbial communities in complex environmental samples.

Executive summary:

The LANL SFA program in Terrestrial Microbial Carbon Cycling aims to inform climate modeling and enable carbon management in terrestrial ecosystems. To achieve these aims, our program develops and uses community genomics approaches to discover widespread biological processes that control carbon storage and release in temperate biome soils.

Our progress in use of metagenomic techniques to detect and describe the composition of microbial communities in complex environmental samples can be summarized in six aspects:

1) We capitalized on technology advances in metagenomic sequencing and data analysis to increase (10 to 100-fold) our depth of sampling of microbial communities and to gain better insights (e.g. [2-12]).
2) We tested and developed technologies (e.g., PCR primer sets and various classification tools) for metagenomic sequencing and analysis. (e.g. [4, 13-17])
3) We enhanced databases that are a foundation to interpret metagenomic data to describe the taxonomic and functional gene composition of fungal and bacterial communities (e.g. [14-16, 18-24]).
4) We used metagenomic data from microbial communities in complex environmental samples to guide isolation/discovery of priority bacteria and fungi widely sought by the international community of environmental microbiologists [1, 21, 22, 24-26].
5) We used metagenomic techniques to substantially increase knowledge of the composition of fungal and bacterial communities in 13 terrestrial ecosystems across the U.S. responding to drivers of ecosystem changes in carbon cycling (elevated atmospheric CO_2, elevated inorganic nitrogen deposition, drought, land-use changes), including variation of microbial communities with soil depth and seasonal effects. (e.g. [3, 4, 6-8, 10-12, 23, 27-34]).
6) We used insights from our application of metagenomics techniques in terrestrial ecosystem experiments to improve our strategy to achieve the long-term aims of our SFA program. From this strategic shift, we are currently completing analyses of metagenomic data describing bacterial and fungal communities from over 400 field samples from 10 western US states and a related set of 500 litter decomposer communities—a prioritized subset from more than 1600 microcosms from experiments in our laboratory—to gain insight into microbial control of carbon flow (publications in prep).

Background

Metagenomics techniques are broadly categorized as either targeted or shotgun. Targeted metagenomics involves amplification and sequencing of a chosen gene to determine either "who" is in a community or what they can do (i.e., how many forms are present of an enzyme

that catalyzes a key step in an environmental process of interest, like carbon or nitrogen cycling). Crude forms of targeted metagenomics began in the 90's. Shotgun metagenomics involves sequencing a bit of everything in the total pool of DNA that can be extracted directly from an environmental sample. Shotgun metagenomics began as a crude but feasible practice after 2007.

(1) Capitalizing on technology advances to improve community analysis.

Our research program began 10 years ago when shotgun metagenomics for complex environmental samples was first emerging, and its use for comparison of ecosystems was mostly conceptual. Analysis pipelines did not exist. Even the concept of sequencing replicate samples to enable statistical analyses of experimental treatments was novel---an early battle our program fought with managers at JGI—because of the cost and sequencing capacity needed. Our first use of targeted metagenomics to examine microbial community (i.e. bacteria and fungi) responses in six DOE climate change field experiments examining long-term effects of elevated atmospheric CO_2 used Sanger sequencing of clone libraries and was the first-of-its-kind attempt to find common microbial responses to climate manipulations in multiple ecosystem experiments by "large-scale" sequencing [4]. To increase the depth of sampling microbial communities in targeted or shotgun metagenomics, we progressively capitalized on 454 Pyrosequencing when it became feasible through JGI [4, 9-12, 23, 32, 33, 35] and subsequently iterations of Illumina sequencing chemistry and instrumentation [2, 6, 8]. Similarly, we progressed from use of our own custom software for processing targeted metagenomic data [4] to use of ever-more powerful and publicly available metagenomic and ecological analysis tools (e.g., [2, 7-9, 31, 33, 36]). An ongoing challenge with metagenomic data is the difficulty of comparing data over time and among laboratories owing to data variability arising from DNA preparation procedures, DNA sequencing runs, DNA sequencing platforms, and different users. Like the broader research community, we continue efforts to increase data quality (manuscript in prep), standardize methods, and identify best-practices to improve metagenomic data and associated research findings.

(2) Testing and developing technologies for metagenomic sequencing and analysis.

Our efforts to expand or improve options for metagenomic sequencing primarily focused on PCR primer sets and classification tools for targeted metagenomics [4, 13-17]. Our program was a leader in simultaneously examining fungi and bacteria in environmental samples [4, 12]. Historically, researchers focused on one group or the other, not both—a bias that still continues although there is now a rapidly growing number of researchers that have adopted our approach of examining both groups. For fungi, our early studies focused on cellulolytic fungi by analysis of cellobiohydrolase I gene sequences (cellobiose is a disaccharide released as cellulose is hydrolyzed) and the broader fungal community by using the large subunit rRNA gene (LSU) for taxonomic analyses [12, 23, 35]. Although the LSU gene does not provide as much resolution at the species level as the internal transcribed spacer (ITS) region, which has been the convention target in mycology, the LSU gene uniquely enables reconstruction of phylogenetic trees and the use of *phylogenetic* diversity metrics (e.g. Faith's PD) that have emerged for ecological analysis of communities.

Fungal LSU primers for Illumina sequencing. Previously, the most commonly used primers to target the fungal ribosomal large subunit (LSU) was the LR0R-LR3 primer pair,

which gives an amplicon (~650 bp) that has been outside the range of existing Illumina paired-end sequencing lengths. To overcome this limitation, we developed a primer to amplify a shorter fragment of the fungal LSU, targeting the D2 hypervariable region [16]. We compared community metrics of environmental samples obtained using the new primer and two other commonly-used rRNA regions, the D1-D2 LSU and the internal transcribed spacer 2 (ITS2). The new primer pair was found to have only slight bias within the Fungi (primarily in the Glomeromycota), comparable to the standard D1-D2 LSU fragment, and was more taxonomically accurate than the ITS2 fragment. The new primer increases targeted metagenomic sequence quality by enabling overlap of forward and reverse reads. The primer set remains a central tool in our program (e.g., [5]).

qPCR assay design platform. Possibly the most sophisticated PCR primer assay design platform in existence was developed over the course of a decade at LANL through other programs to handle the most challenging design problems. To augment our SFA program, we further improved the primer design platform, achieving a 15-fold increase in number of useful candidate assays, and we used the platform to develop qPCR assays to validate findings from our ecosystem metagenomic studies [4, 37].

Increased return on investment from Illumina MiSeq. The standard protocol from Illumina for targeted metagenomic sequencing wastes about 30% of the sequencing capacity on sequencing a bacteriophage genome. We launched a practice of replacing the virus genome with new bacterial or fungal genomes of interest in order to increase return on investment, acquiring drafts of 9 fungal and 11 bacterial genomes. We are currently using this strategy to acquire draft genome sequences of 15 fungal species that are relevant to metagenomic studies of carbon cycling terrestrial ecosystems.

Classification of dormant & active bacteria. We used modeling and simulations to improve interpretation of ribosomal RNA/DNA ratios from metagenomic data [17]. Ribosomal RNA/DNA ratios from metagenomic data have been of interest to microbial ecologists as a value-added way to identify the active bacterial species in complex environmental communities. Our simulations of targeted metagenomic data showed the potential for substantial misclassification of active populations as dormant, and we used insights from our simulations to suggests ways of using metagenomics to acquire ribosomal RNA/DNA ratios that provide more robust conclusions [17].

(3) Enhancing databases that are the foundation to interpret metagenomic data.

Fungal rDNA database improvement. When our program began, phylogenetic databases for analysis of fungal rDNA gene sequences were extremely small and immature compared to those for bacterial rDNA. Public reference sequences for fungal taxa were either very limited in scope or were not comparable because the reference sequences represented completely different genomic markers. The 20,000-member sequence collection of fungal LSU sequences we obtained from the six ecosystems with elevated CO_2 studies represented one of the largest high-quality

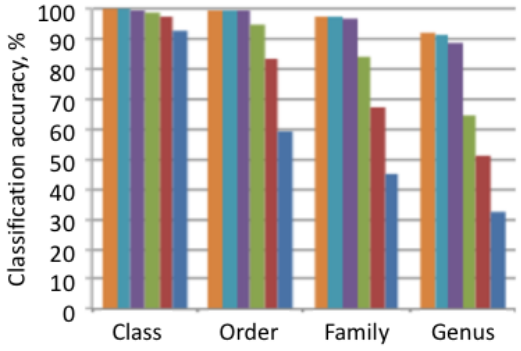

Figure 1. Classification accuracy as a function of taxonomic hierarchy and fragment sequence length from primer LR0R left to right: 400, 300, 200, 150, 100, 75 bp. (from [19]).

environmental fungal rDNA collections at the time [12]. The sequence collection boosted development of a fungal LSU classification database.

We quantified the accuracy of the LSU region for fungal identification (Fig. 1) and supplied the Ribosomal Database Project (RDP, Michigan State University) with a hand-curated LSU classifier [19] to which we have continued to make improvements. We compared the LSU and ITS for community assessment [20], and we also participated in release of a more comprehensive ITS database [15].

Functional gene database improvement. Our collection of *cbh*I and *nif*H acquired through targeted metagenomic studies of these marker genes for cellulose degradation and for nitrogen fixation [3, 9, 23] contributed to the functional marker gene databases hosted by the Ribosomal Database Project.

(4) Using metagenomic data to guide isolation/discovery of priority bacteria and fungi.

We obtained and genome-sequenced an isolate of the cyanobacterium *Microcoleus vaginatus*—the dominant photosynthetic cyanobacterium in arid grassland biocrusts [21]. We also sequenced the genomes of isolates representing five heterotrophic genera that occur in biocrusts to provide insight into possible interactions between *M. vaginatus* and its bacterial partners.

Metagenomic data from our arid land research sites guided isolation of under-represented Actinomycete bacteria. We successfully cultured isolates representing eight of the most dominant Actinomycetales families in arid land soils [24]. We characterized the ability of a large panel of isolates to catalyze extracellular degradation of complex polysaccharides (crystalline cellulose, carboxymethyl cellulose, xylan, chitin, pectin and starch) and express peroxidase and laccase activity. Exopolysaccharide degradation was a widespread capability among the Actinomycetales, but degradation of specific substrates tended to be phylogenetic clustered [24]. We obtained genome sequences from eight diverse, under-represented Actinomycetales, enhancing representation of Actinomycetales and their functional capabilities in the JGI-IMG public database.

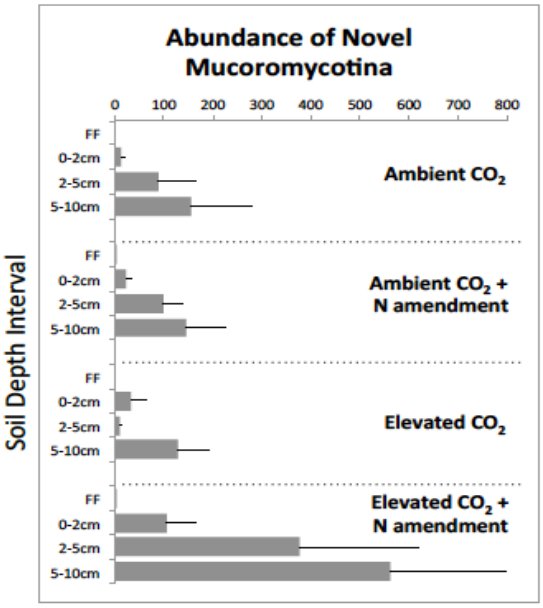

Figure 2. Abundance of novel Mucoromycotina rDNA sequences across four depth intervals in a pine forest exposed to over 10 yrs of elevated CO_2 and/or 6 yrs of N amendment. Note the increase in abundance with depth and with the combination of

We used metagenomic data from our pine forest N-deposition study to guide isolation of Mucoromycotina (fungi) isolates for genome sequencing [12, 22]. The metagenomic data showed that certain yeast-form Mucoromycotina (Zygomycota) were abundant in the lower, mineral soil horizon of the pine forest soil, and these fungi responded positively to N deposition and to elevated CO_2 conditions (Fig. 2). The sequence identities to reference sequences were low, suggesting these were novel fungi. Using a combination of sieving, dilution, and creative selective media, our program acquired a number of

isolates in this group. We characterized and sequenced an isolate identified as *Bifiguratus adelaidae*, one of the international research community's 'top 50 most wanted' fungi [22].

(5) Increased knowledge of fungal and bacterial communities in 13 terrestrial ecosystems across the U.S. responding to drivers of ecosystem changes

The 13 terrestrial ecosystems we examined (Fig. 3) included 6 ecosystem studies of the effects of elevated atmospheric CO_2 and 7 ecosystem studies of the effects of N deposition (i.e. increased inorganic nitrogen). Elevated atmospheric CO_2 and N deposition are both anthropogenic perturbations that are increasing and are altering ecosystem processes. Illustrative examples of our progress using metagenomics to increase knowledge of terrestrial ecosystems are summarized below; the examples provide context for the major shift in our research strategy described in the final section of this report.

Figure 3. Major biomes and approximate ecosystem locations (field experiment sites, ●) that we examined.

Our program began (2009-2012) with a study of the impacts of over 10 yrs of elevated atmospheric CO_2 on soil ecosystem processes and the microbial communities responsible for those processes in long-term (~10 yr) field studies led by other DOE-funded research teams using a free air CO_2 enrichment (FACE) or open top chamber (OTC) approach in six temperate ecosystems. A central objective of our project was to begin developing soil metagenomics as a research tool and apply this unique tool across ecosystems to gain insight into soil microbial responses to future climate regimes. The central hypothesis was that common microbial responses occur among different ecosystems exposed to climate forcing. Identification of common responses and the underlying microbial processes could inform climate models projecting carbon cycling over centennial time scales. We published over 14 manuscripts from this work, comparing the responses of soil fungal and bacterial communities to elevated CO_2 *across* ecosystems and the specific responses *within* each ecosystem individually in the context of other experimental variables examined at each site (N amendment, ozone, plant association, soil depth).

Among ecosystems: soil fungal community responses to elevated to elevated CO_2. Elevated atmospheric CO_2 generally increases plant productivity, increasing the availability of cellulose to microbial decomposers and the plant demand for nitrogen. Soil fungi, as primary cellulose degraders, are likely to be affected by the ecosystem changes that occur under elevated atmospheric CO_2. We examined treatment responses of the soil fungal community through parallel targeted metagenomic surveys of the fungal LSU rRNA gene in six ecosystems, aided by our fungal LSU classification tool [12, 19]. The fungal communities were typically more spatially variable than the bacterial communities within each ecosystem, and statistically significant changes in fungal community biomass, richness, or composition were ecosystem-specific, with no discernable common pattern [12].

To investigate the impacts of ecosystem type and elevated atmospheric CO_2 specifically on cellulolytic fungal communities, we sequenced 10,677 *cbhI* gene fragments encoding the catalytic subunit of cellobiohydrolase I, from five of the FACE sites [35]. The *cbhI* composition

of each ecosystem was distinct and the magnitude and direction of response to elevated CO_2 differed by site. The arid shrubland was one of the most responsive, with major shifts in abundant *cbh1*-containing fungal taxa. Less than 1% of the *cbhI* sequences could be classified to the family level indicating that cellulolytic fungi *in situ* are likely dominated by novel fungi or known fungi that are not yet recognized as cellulose degraders. In the arid shrubland creosote bush soils, *cbhI* gene richness was significantly higher after a decade of exposure to elevated CO_2 compared to ambient CO_2 conditions. In arid shrubland biocrusts, richness was not altered, but the relative abundance of dominant operational taxonomic units (OTUs) in the biocrusts exposed to long term elevated CO_2 was significantly shifted. While the observed changes clearly indicate a response to altered ecosystem behavior, we were unable to infer functional consequences of the various changes documented in each ecosystem.

Within ecosystems: Biocrust and shrub root zone responses to elevated CO_2 at the arid shrubland FACE site. Like other ecosystems in the drylands biome of the US west (Figure 3), the arid shrubland ecosystem at the Nevada Test Site (NTS) in the Mojave Desert is characterized by extremely patchy plant cover. The dominant shrub, *Laccaria tridentata* (creosote bush), and native grasses are widely spaced, and between the patchy plant "islands" cyanobacteria-dominated biocrusts cover the soil surfaces. In creosote root zones, fungal community richness was lower in elevated CO_2 soils than in ambient CO_2 soils [3, 12, 32]. The reverse was true for bacterial richness, suggesting the plant response to elevated CO_2 had altered root zone C and consequently, the relative activities of fungi and bacteria. A large proportion of the bacterial richness increase was attributable to the Actinobacteria. However, the functional consequences of the observed changes in fungal and bacterial communities for overall ecosystem carbon cycling remain unknown.

In the biocrusts, we predicted that photosynthetic cyanobacteria would respond positively to long term elevated CO_2, analogous to plant responses in forested ecosystems. Contrary to our prediction, our surveys employing cyanobacterial-specific qPCR, 454 rRNA sequencing, and shotgun metagenomes all suggested that cyanobacterial biomass decreased in elevated CO_2 soils (Figure 4). The decrease in cyanobacterial biomass was nonspecific and was manifest across diverse cyanobacterial groups. Metagenomic sequencing showed that genes related to oxidative stress were differentially abundant between the elevated CO_2 treatment and controls, suggesting that elevated CO_2 selected for cyanobacteria with differential abilities to deal with reactive oxygen species and may explain the decrease in cyaonobacterial biomass [32].

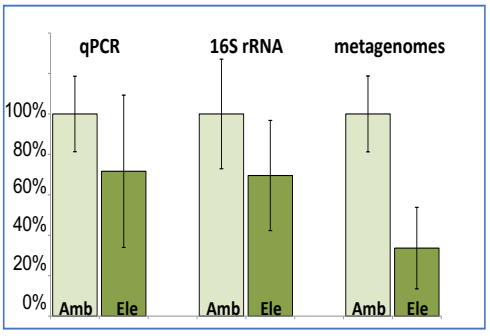

Figure 4. Reduction in cyanobacterial abundance under elevated CO_2. The % reduction in biomass (qPCR) or representation in rRNA or shotgun metagenomes in replicate elevated CO_2 soils is displayed. Error bars represent the standard deviation of the mean.

High-level summary findings from our metagenomic studies of 6 ecosystems under elevated CO_2 and other factors:

- The fungal and bacterial communities each differed significantly among the ecosystems [3, 4, 12, 35].

- The biomass and composition of the fungal and bacterial communities was strongly influenced by local soil geochemistry and also by soil depth across shallow (cm) horizons in all of the natural ecosystems [3, 4, 9, 10, 12]. Responses to elevated CO2 in the fungal and/or bacterial communities were identified in each of the field experiments. Although some common trends were distinguished, most responses were ecosystem-specific and were usually secondary in magnitude to other experimental factors, such as N amendment, ozone exposure, soil depth, or plant root association. Often, elevated CO2 responses were interactive with other experimental factors.
- In the one FACE site where time-course sampling was feasible (a mesic pine forest in North Carolina), we found no evidence of seasonal differences in fungal community composition or N amendment response [6].
- Across sites, the diazotrophic bacterial community was much more diverse than previously described [3].
- Use of targeted sequencing approaches, for examples the bacterial rRNA gene, the fungal cellobiohydrolase gene (cbhI), or the bacterial nitrogenase dehydrogenase gene (nifH) provided higher resolution discrimination among communities and treatments than shotgun metagenomes, likely due to higher sampling efficiency and ability to identify and bin sequence reads [3, 9, 23].
- The shotgun metagenomes differed greatly by soil type/region when analyzed using functional gene assignments (SEED subsystems within MG-RAST), or with gene-independent kmer analyses but could rarely discern treatment differences within a site [9, 10].

Overall, our metagenomic studies of ecosystems exposed to elevated atmospheric CO_2 showed ecosystem-specific responses. The difficulty of finding common responses and the inability to determine the functional significance of individual responses began to illuminate a challenge in our approach to use of metagenomics to inform climate models. We transitioned to examining microbial responses in 8 ecosystem studies of the effects of N deposition (i.e. ecosystem exposure to elevated levels of inorganic nitrogen such as ammonium or nitrate; the field experiments were led by external research teams). Because N deposition tends to perturb soil microbial communities more strongly than elevated CO_2, we reasoned that discovery of common patterns of microbial response to N deposition among ecosystems may be more tractable. For illustration, some findings from a pine forest ecosystem and arid ecosystems are summarized below.

Soil microbial responses to N amendment in shallow soil horizons in a pine forest ecosystem. We performed metagenomic studies in different years at the North Carolina pine forest experiment examining long-term exposure to N amendment [1, 12, 23, 36]. Our studies showed (a) dramatic differences in soil geochemistry across centimeter depth intervals from forest floor to mineral A horizon, (b) corresponding, very large differences in fungal biomass and composition across the cm depth intervals, (c) strong impacts of 5 yr of N amendment on horizon

geochemistry, especially in the forest floor and O horizons, (d) major changes in fungal species richness and composition in response to N amendment that were horizon-specific (Fig. 5). These studies revealed major phyla-level shifts in community structure that may influence plant mycorrhizal interactions and decomposition processes in this forest under N amendment, and it demonstrated the complexity of responses over small depth intervals. The latter finding suggests the multitude of prior studies (including our own) that had, for decades, assessed soil processes in a homogenized 0-10 cm or 0-30 cm sample may have obscured many microbial responses to N amendment that are only apparent over smaller depth intervals.

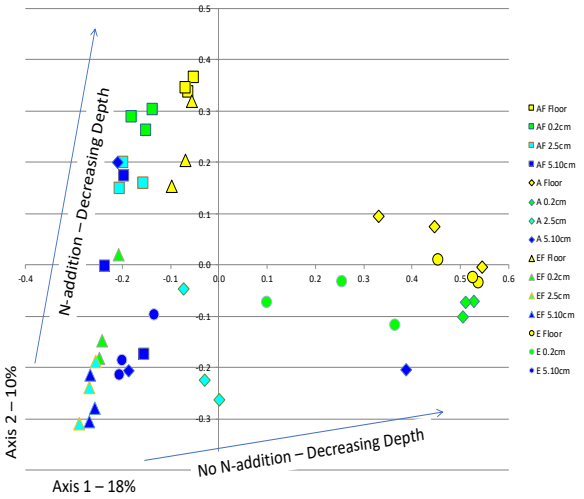

Figure 5. Illustration of fungal community composition shifts across 3 soil horizons in the pine forest (yellow – forest floor; green – O horizon; blue – A horizon) in soils with and without N amendment. [1]

Soil microbial responses to N amendment in aridland ecosystems. We assessed plant, soil chemistry, fungal and bacterial community responses to two years of N deposition in three aridland ecosystems (two in Nevada and one in Utah). Responses were measured in discrete microenvironments (e.g., biocrust at 0-0.5 cm depth and bulk soil 0-10 cm depth in interspaces and under plant canopies) [38]. Collectively, metagenomic data revealed extensive variation in microbial community responses over depth profiles, between biocrusts and plant islands, and between ecosystems. Whereas non-metagenomic assays indicated a common trend among ecosystems in microbial carbon use efficiency under N amendment, we have not yet been able to identify a corresponding metagenomic signature that reflects a common physiological process among ecosystems.

Overall, our metagenomic studies of ecosystems exposed to elevated N amendment documented numerous, highly variable and location specific microbial responses within and among ecosystems. While these studies build a useful baseline of foundational knowledge, they also underscore the difficulty of informing climate models via the application of metagenomic techniques in the context of the conventional experimental approach to understand the contribution of microbial processes to ecosystem function.

(6) Insights improving our strategy to achieve the long-term aims of our SFA.

We usefully exploited our experience applying metagenomic techniques to ecosystem studies to develop a new research strategy. Our past experience highlighted a common, fundamental challenge: documenting microbial responses to perturbations is fairly easy with metagenomic techniques, but identifying which responses matter is extremely difficult. Studies designed to examine ecosystem response to changes in physical or chemical variables often find numerous microbial community changes/responses. Many responses are likely cosmetic. Which are functionally consequential? Which responses reflect mechanisms that are not already captured by physicochemical or plant phenomena embedded in current soil carbon models?

Our new strategy emphasizes discovery of microbial communities that represent substantially different (and measurable) functional states *under the same environmental conditions,* not

perturbed versus unperturbed conditions. This approach shifts the focus from discovering responsive populations to directly discovering populations and processes that cause specified functional states.

Our revised strategy requires sampling a large number of microbial communities and measuring the function of each one *under the same environmental condition* in order to identify functional states of interest. Once the functional states are delineated, metagenomic techniques

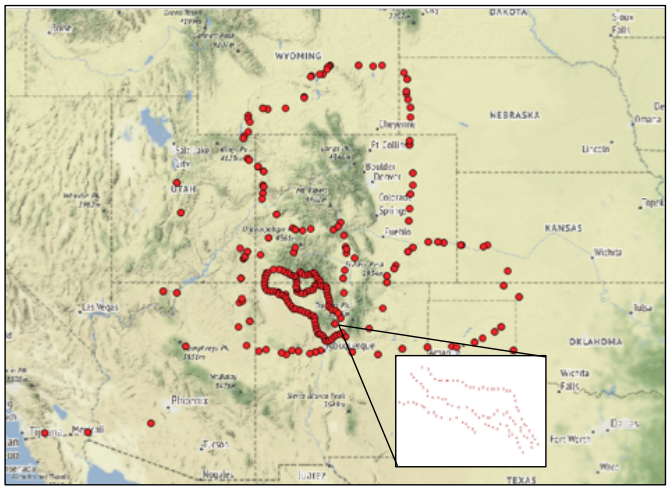

Figure 6. Soil sample locations spanning 10 states in the US west.

enable the search for the common features or processes that underpin each state. Towards this end, we collected over 400 soil samples from locations representing different spatial scales in the drylands biome of the US west (Fig. 6). We have and are continuing to use targeted metagenomics to characterize the native fungal and bacterial communities in soil samples of particular interest (manuscripts in prep). Our prior and current work with soils in this biome as well as work by others has shown that Ascomycota are the dominant fungi (65-95%) in these drylands soils and Actinobacteria one of the most abundant bacterial phyla.

To measure and delineate functional states, we performed plant litter decomposition experiments in simple laboratory microcosms, measuring CO_2 and dissolved organic carbon arising from litter decomposition over 30 days. Our functional states of interest are delineated by DOC abundance. The two states are described as high and low DOC, respectively, and the means for the two states differ substantially (e.g., ~2-fold).

We are in the process of applying metagenomic techniques and analyzing data to characterize the fungal and bacteria decomposer communities in 500 microcosm samples, down-selected from a total of 1600 microcosms for which we have measured CO_2 and DOC. We have performed decomposition experiments with litter from 4 different types of plants in order to discover common bacterial processes that can create different functional states regardless of plant litter type. The average composition of bacterial and fungal communities on 3 of the plant litter types is illustrated below (Fig.7). As expected, the average composition of the decomposer communities varies by plant litter type, reflecting the importance of litter chemistry in shaping decomposer communities—a phenomenon demonstrated by many researchers.

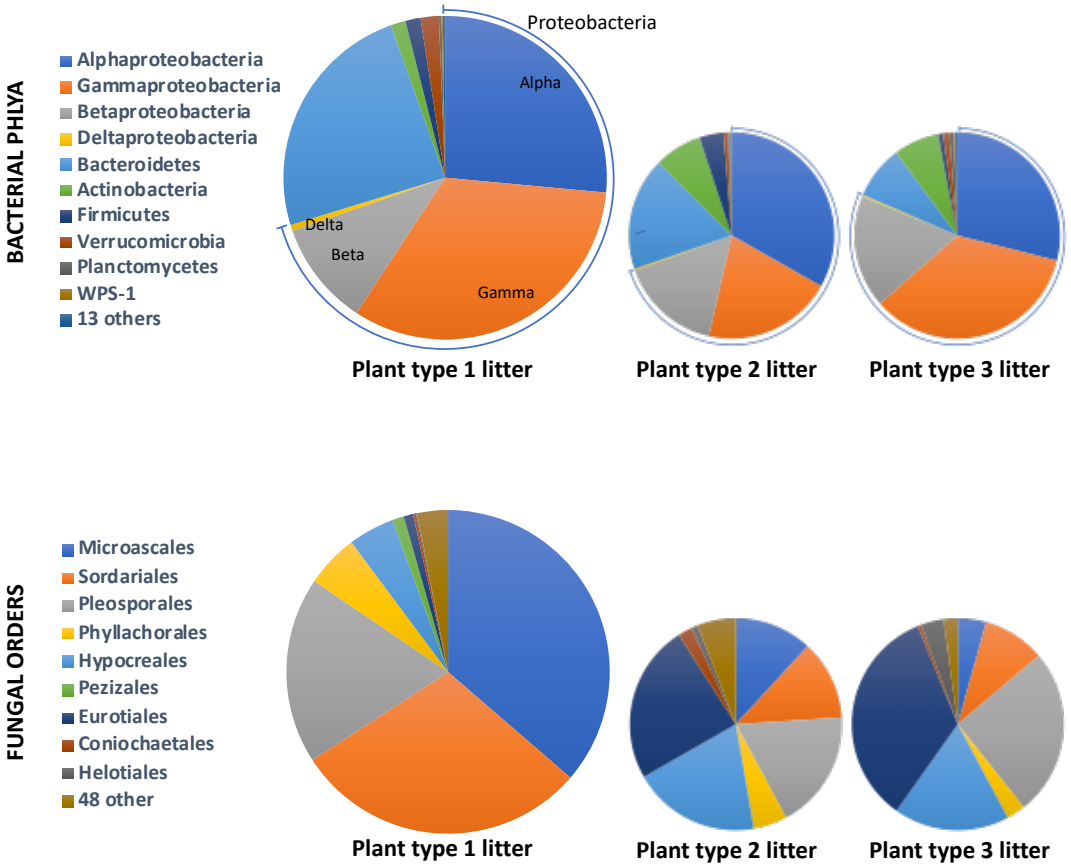

Figure 7. Illustration of the average composition of microbial decomposer communities that assembled on 3 different plant litter types over 30 days after inoculating microbial communities from soil samples into the microcosms (manuscripts in prep). The average composition was determined from 50 to 128 communities for each litter type.

Our interest is the common taxonomic and/or metabolic features that underpin the high and low DOC functional states among litter types. We have found that a substantial fraction of the taxa shown in Fig. 7 exhibit similar shifts in relative abundance between the high and low DOC functional states among litter types. For example, for each litter type, the Deltaproteobacteria was >3-fold higher, on average, in communities representing the low DOC functional state compared to the the high DOC functional state (Fig. 8). These data are a promising indication that our revised strategy will provide insights to biological processes that may broadly influence carbon cycling.

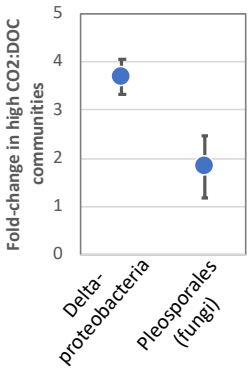

Figure 8. Example of taxa exhibiting consistent patterns among litter types (manuscripts in prep).

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